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14. ABSTRACT

Mutations in the BRCA1 checkpoint gene results in aneuploidy and an increased risk of breast cancer. The yeast RAD9 protein has similar functions and sequence motifs as BRCA1 and we proposed to identify haploinsufficient mutations at a second locus that alters the chromosome loss rate of our rad9-/- diploid strains. We created a rad9Δ/Δ strain both a qualitative (sectoring colonies) and quantitative assay (canavanine resistance) sensitive enough to detect the increase of heterozygous mutations on chromosome loss rates. We analyzed 30,000 insertional mutants and obtained 400 independent insertions that reproducibly alter CF loss rate. By FA, 40% of this group show statistically significant increase (3-10 fold) in mutation rate. We identified the insertion site for 300 mutants and gene ontology analysis of the 225 independent insertions reveals a statistically significant over-representation for Cell Cycle process and Chromosome location and under-representation of the Protein Synthesis process. Recreation of precise gene deletions in wild-type and rad9Δ/Δ backgrounds verifies the instability phenotype and distinguishes heterozygous mutations that are specific modifiers of rad9 mutant strains from those that cause genomic instability independently.

15. SUBJECT TERMS
BRCA1, genomic instability, modifier genes, genetic screens

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Introduction:

The *BRCA1* breast cancer susceptibility gene was identified after a massive positional cloning effort focused on families containing at least four cases of breast cancer[1]. Once the gene was identified testing for mutations became clinically available. Subsequent analysis of women who carry a *BRCA1* mutation has demonstrated that the penetrance (the likelihood one will develop cancer if you carry a mutation) is quite different depending on the population studied. Estimates of penetrance from the original Breast Cancer Linkage Consortium were as high as 80% for breast cancer. Other studies using unselected populations lead to estimates of 30-50%. Similarly, the risk for ovarian cancer is dependent on how the study is performed. One explanation for these different cancer risks is the potential for other genetic loci (termed modifier genes) to influence cancer risk.

There are several approaches to identify specific modifier loci in individuals who carry *BRCA1* mutations. The most commonly used approach is to directly test loci that influence cancer risk in the general population or have a biologically plausible reason to hypothesize an influence on BRCA1. For example, specific alleles of a VNTR polymorphism in *HRAS1* influences ovarian cancer risk in *BRCA1* carriers. The latter approach is exemplified by studies of genes involved in the response to DNA damage. For example, a polymorphism in the 5' UTR (untranslated region) of the *RAD51* gene influences cancer risk in women who carry *BRCA2* mutations but not *BRCA1* mutations. More recently, genome-wide studies for genetic modifiers have been initiated. The PI of this proposal is participating in a multi-institutional study through the Cancer Genetics Network to collect DNA samples and clinical data from hundreds of women who carry *BRCA1* or *BRCA2* mutations. Genome-wide scans will be used in a modified linkage approach to identify loci that influence the age of cancer diagnosis in these women. These linkage methods are likely to be successful but are very large-scale efforts that when successful still implicate a locus that may contain dozens to hundreds of genes. No straightforward methods are available to sort the genes in the region for potential to carry an important polymorphism. Therefore, in this Exploratory Award we proposed to develop an innovative approach to use the simple eukaryote, *Saccharomyces cerevisiae,* to rapidly screen the genome for mutations that modify genomic instability. The genes identified in this study can then be candidates for focused epidemiologic studies.

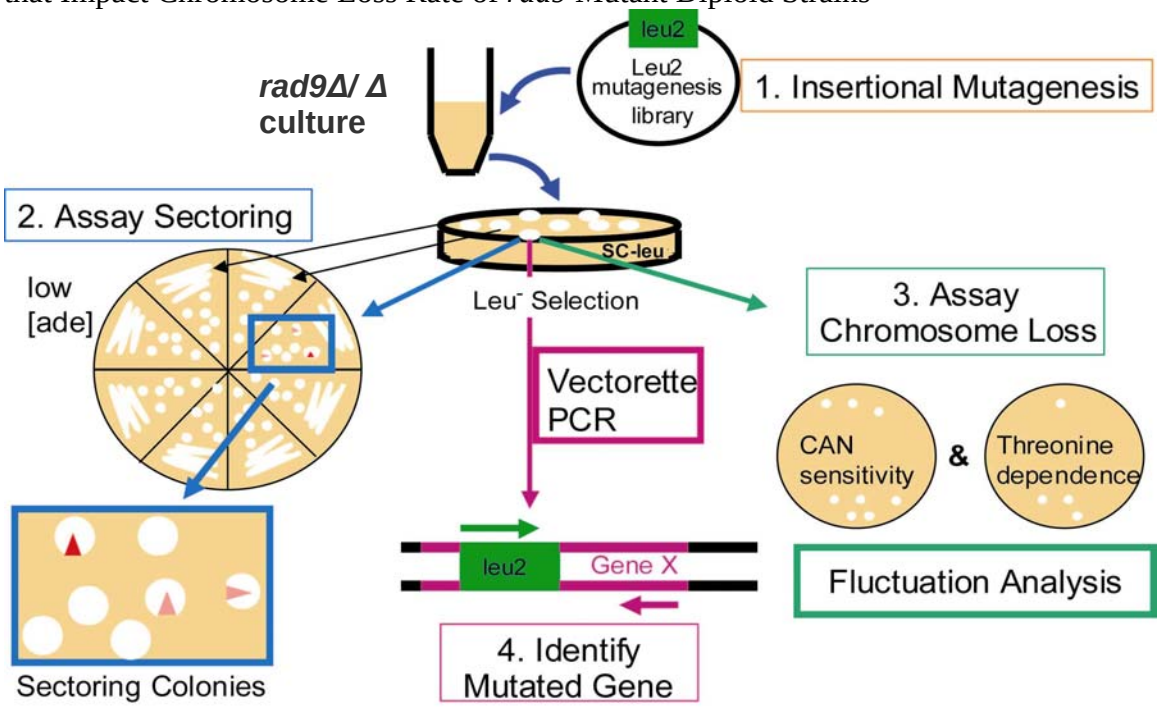
Though no direct ortholog of the human BRCA1 gene has been identified in the *S. cerevisiae* genome the human BRCA1 protein is similar to the budding yeast Rad9 protein in several ways[2]. *rad9* mutant strains and *BRCA1* mutant cells have defective G2 checkpoint responses and are sensitive to several forms of DNA damage including ionizing radiation, alkylating agent exposure and UV radiation. In addition, the ATM (mutated in ataxia telangiectasia) kinase phosphorylates BRCA1 in response to DNA damage just as Mec1, a *S. cerevisiae* ATM homolog, phosphorylates Rad9 in yeast. Furthermore, both BRCA1 and Rad9 proteins contain a C-terminal tandem repeat of the BRCT (Brca1 carboxy terminus) motif. Mutating the Rad9 BRCT domain results in phenotypes similar to those observed in *rad9Δ* strains confirming that it is an important functional domain. Mutations in the BRCA1 BRCT motif results in an increased risk of breast cancer in women.

As first reported by Weinert and Hartwell[3], exponentially growing *rad9Δ* strains show a 10- to 20-fold increase in the spontaneous rate of chromosome loss. Recently, Klein and colleagues directly compared chromosome loss rates among a series of checkpoint defective *S. cerevisiae* strains. They found that *rad9Δ/Δ* strains had a chromosome loss rate of 2.0×10^{-5} compared with 2.0×10^{-6} for a comparable wild type strain. Based on this conservation in function, we proposed to develop and perform novel genetic screens in yeast that will allow rapid identification of loci which when haploinsufficient modify the chromosome loss phenotype (either increase or decrease) of a *rad9Δ/Δ* diploid strain. These genes will serve as candidate modifier loci of genomic instability resulting from loss of *BRCA1* function in mammary cells.

Body

In the sections below we document the progress towards completion of the tasks in the original statement of work. For each section we have included our progress towards completing that task. Figure 1 gives an overview of the strategy we developed over the course of this Exploratory Award to complete the genetic screen.

Figure 1 – Overview of the Genetic Screen Devised to Identify Heterozygous Mutations that Impact Chromosome Loss Rate of *rad9* Mutant Diploid Strains



Statement of Work:

Task 1 – Engineer *rad9Δ/Δ* strain for rapid and visual selection of mutants which alter chromosome loss rate using repressive chromosome fragment.

a. Mutate endogenous *ADE*2 gene to create *ade2-101* ochre mutation and introduce chromosome fragment (CF) containing ochre suppressor SUP11 gene in selected diploid strains.
b. Determine sectoring rate for parental strains and best methodology for visualization of sectors.
c. Utilize mTn-lacZ/LEU2-mutagenized library to create insertional mutants, each carrying one mutation, collectively comprising mutations throughout the genome.Streak 30,000 insertional mutants, giving 95% coverage of the genome, and visualize 200 colonies per mutant counting sectoring phenotype based on loss of the CF.

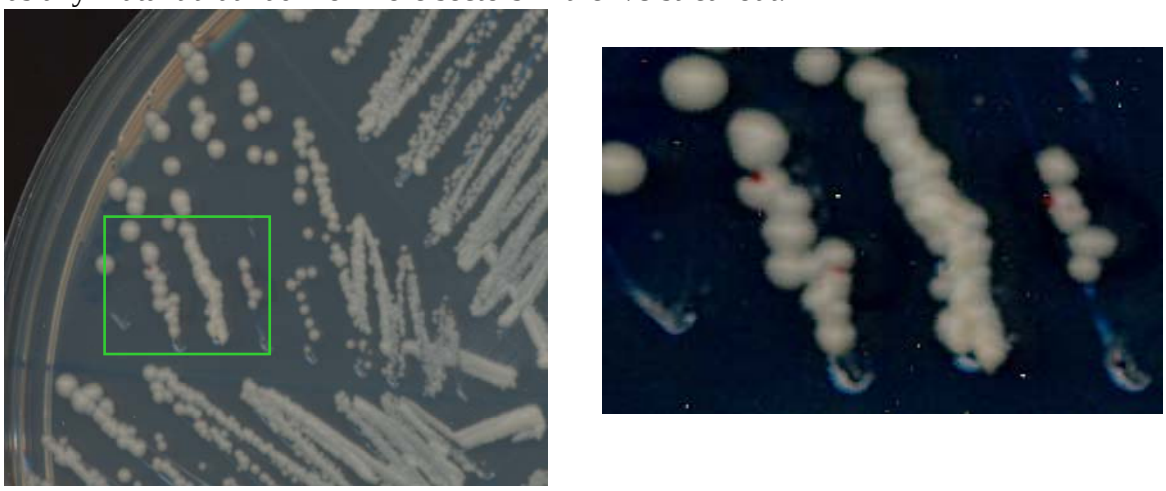
Progress

Task 1a. In order to use the *ADE2* marker, we engineered *rad9*Δ/Δ diploid strain to contain an *ade2*-101 mutation which is an ochre mutant that is capable of being suppressed by a *SUP11* tRNA gene contained on a chromosome fragment (CF). Dr. Vicki

Lundblad kindly supplied us with yeast strains containing a CF with one copy of the *SUP11* gene. In the absence of the *SUP11* gene, strains carrying an *ade2-101* mutation will cause a yeast strain to build up adenine precursors and turn a red color. When a diploid yeast strain carries two CFs, and thus two copies of *SUP11,* the mutation and subsequent build up of precursors are suppressed and the yeast returns to a wild-type white color. However the suppression via SUP11 is leaky and thus carrying only one copy of SUP11 (one CF) in a diploid *ade2-101/ade2-101* background results in a pink colored yeast colony. Any genomic instability resulting in CF loss can be visualized in this system as the appearance of pink or red sectors on a white colony due to lack of suppression of the *ade2-101* mutation. We then utilized this system to visually quantitate CF loss rate by counting colonies that sector, indicating a loss in the copy number of the SUP11 gene. (See example of assay in Figure 2.)

Figure 2 – Left: Example of Streak Out of a Mutant Strain in order to Monitor for Loss of the Chromosome Fragment. Right: An Enlargement of the Area in the Green Box Demonstrating Four Red/Pink Sectored Colonies.

Task 1b. We carried out a number of experiments to determine the expected level of CF loss rate in the parental strain and the variance in this measurement. After a number of trials we determined that if we streak out a strain on one eigth of a 10 cm petri dish we expect to see fewer then 1 sectored colony (out of about ~200 colonies in that area of the plate). We set this level for mutant strains to be further characterized in subsequent tasks as any mutant that had 4 or more sectors in the 1/8 streakout.



Task 1c. Dr. Hannah Klein has graciously supplied us with *rad9*Δ/Δ yeast strains carrying a marked chromosome V for assaying mutation rates[4]. Chromosome V carries the CAN1 gene which encodes the yeast arginine permease, responsible for uptake of arginine and also capable of transporting the arginine ortholog and toxic drug L-canavanine into the cell. Strains that are CAN1/can1-100 are sensitive to canavinine, but losing the wt copy of the gene confers resistance to the drug as it is no longer taken up into the cell. Loss of the wt copy can occurs through loss of the chromosome or recombination with the mutant allele, additively these compose the mutation rate of the cell.

The method used to measure chromosome loss rate is termed fluctuation analysis (FA). This allows one to determine actual mutation rate (as opposed to mutation

frequency, which reflects both mutation/loss rates as well as the growth of the culture. Optimizing this task in order to efficiently screen the mutant strains derived in this strain took significant work (approximately 6 months). We utilized the Spectrofluor Plus purchased by this grant in order to rapidly quantify the growth rate of the mutant strains. . We have verified that this method allows us to calculate the chromosome loss rate of a given strain within a factor of 2 compared to prior published studies. In addition, the variance of measurements of the same strain done three times is also within a factor of two. Thus, this methodology is sufficient for our goal of identifying strains that have a chromosome loss rate that is about five-fold increased compared with the parental strain.

Task 2 – Perform insertional mutagenesis screen using Synder Library DNA into strains developed in Tasks 1 and 2.

a. Assay for changes in chromosome loss rate.

Progress:

Task 2a: We optimized the method to use the LEU2 insertional Snyder library described in the original application. In order to screen the entire yeast genome we needed to screen approximately 30,000 LEU+ mutant colonies (giving 95% coverage of the genome) onto low adenine plates and counting the number of sectors. See Figure 3 for a breakdown of the sectoring phenotype of the 30,000 mutants.

Insertional mutants in the "consistently high sectorers" group were chosen for furthur study to include identification of the insertion site and quantification of the genome instability causing sectoring via fluctuation analysis.

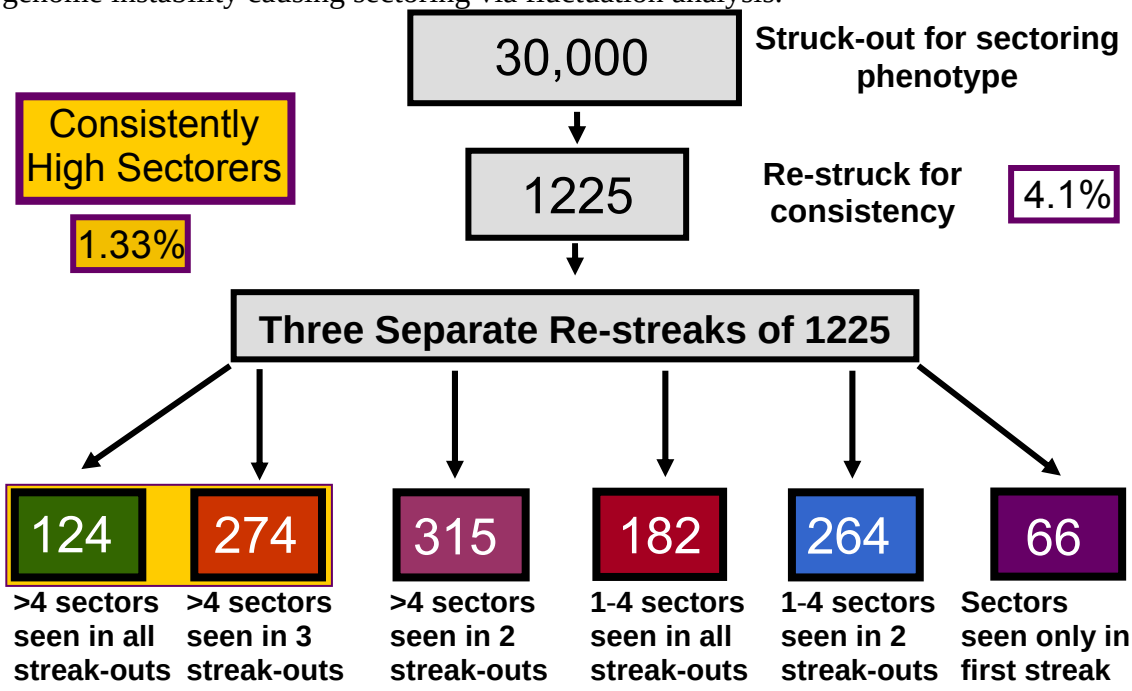


Figure 3 - Sectoring Phenotype of 30,000 Insertional Mutants. After streaking and counting 30,000 mutants, 1225 were found to meet the original threshold of having 4 or more sectors per 200 colonies. These 1225 were then restruck 3 additional times to test reproducibility of this phenotype. Classifications of the mutants were then created based on the sectoring phenotype seen in 4 separate streak-out trials. Two groups (boxes in yellow) were considered "consistently high sectorers" due to repetition of phenotype.

Task 3 – **Utilize Fluctuation Analysis to Quantify Genomic Instability of "Consistenly High Sectorers" Grouping and Identify Insertion Site and Gene Responsible for Measured Instability.**

a. Employ Vectorette PCR and Inverse PCR to Identify insertion site in 398 consistent population
b. Analyze mutation rate of strains with identified insertion sites via fluctuation analysis.

Progress:

Task 3a: Vectorette PCR and Inverse PCR, utilizing 6 different restriction enzyme and multiple double digests for each, successfully identfied insertion site of 300 of the 398 insertional mutation in the reproducible population. Of these 300 sites identified, 171 insertions occurred in novel genes, 54 insertions occurred in "non-coding" regions between genes and the remaining 75 were repetitions of insertions in the aforementioned groups. Table 1 lists the unique insertion sites.

Task 3b: Fluctuation Analysis of 225 insertional mutants, that were classified in the "consistently high sectorers" category, has been completed. Confidence Interval calculations are currently being generated with the collaboration of Dr. Marek Kimmel and Xiaowei Wu at Rice University. Estimates from analysis of forty insertional mutants demonstrate that approximately 40% of the strains are statistically significantly different from the parental non-mutagenized strain, this compares with less then 10% (0 of 10 strains) that were randomly selected from the non-sectoring strains (figure 4).

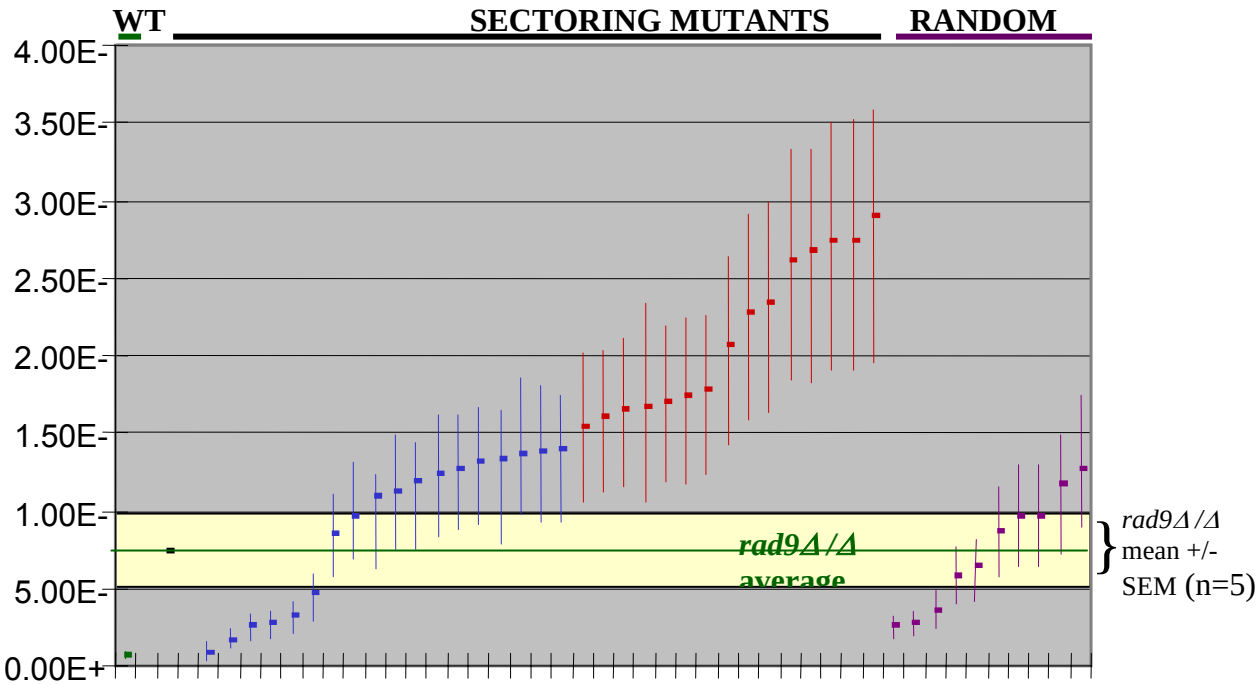


Figure 4. Fluctuation analysis trials on 40 insertional mutants from within the 398 consistently high sectorers demonstrated that 40% of this group show a statistically significant increase in mutation rate (red) over the non-mutagenized rad9-/- parental strains (green). Included in purple on the graph are 10 random insertions, that never sectored, of which none were different from the parental strain.

Table 1 – *Saccharomyces cerevisiae* genes disrupted by insertional mutagenesis which were isolated in the genetic screen described in Task 2.

Gene	Alt name	Function
YDL203C	ACK1	activator of C kinase
APE2	LAP1,YKL157W	involved in peptide metabolism
APL4	YPR029C	inv in vesicle mediated transport
ARO4	YBR249C	catalyzes a.a. biosynthesis
ATG 11	YPR049C, CVT9	transport of aminopeptidases
ATG 13	YPR185W, APG13	reqd for autophagy
ATG26	YLR189C, UGT51	synthesis of sterol membranes
AUS 1	YOR011W	trnsporter of ATP-binding cassette
BFR2	YDR299W	possibly inv in secretion
BPH1	YCR032W	defective lysosomal trafficking
BPT1	YLL015W	transport of unconj bili, heavy metal detox
CDA2	YLR308W	synthesis of ascospore wall
CDC26	YFR036W,HIT3,SCD26	reqd for degradation of anaphase inhibitors
CDC9	YDL164C,MMS8	dna ligase;acts in nucleotide excision repair
CHS3	YBR023C,CAL1,CSD2	chitin synthase III, syn of cell wall chitin
COX13	YGL191W	subunit of cytochrome c oxidase
DBP5	YOR046C,RAT8	ATP-dependent RNA helicase
DBP6	YNR038W	ribosome biogenesis
DBR1	YKL149C,PRP26	RNA lariat debranching enzyme
DID4	YKLO02W,GRD7,REN	sorting of integral membrane proteins
DIP5	YPL265W	transport of L-glutamate and aspartate
DML1	YMR211W	inv in mtDNA inheritance
DPB11	YJL090C	reqd for loading DNA polymerase,checkpt
DUN1	YDL101C	checkpt for DNA damage
DIN7		structural homolog of RAD2 & RAD27
ECM16	YMR128W,DHR1	reqd for rRNA synthesis
ECM7	YLR443W,ZRG15	unknown function
ELG1	YOR144C,RTT110	reqd for Sphase, imp for DNA replication
EMI2	YDR516C	unknown function
ENA1	YDR040C,HOR6,PMR2	salt tolerance
ENA2	YDR039C	salt tolerance
ENA5	YDR038C	sodium transport
EUG1	YDR518W	protein folding
FLO9	YAL063C	inv in flocculation
FOB1	YDR110W,HRM1	DNA replication fork blocking
GCR1	YPL075W	transcriptional activator inv in glycolysis
GDA1	YEL042W	transport of GDP-mannose into Golgi
GPG1	YGL121C	inv in regulation of pseudohyphal growth
GTT1	YIR038C	glutathione transferase activity
HAC1	YFL031W,ERN4,IRE15	specific RNA polymerase activity
HIR3	YJR140C,HPC1	histone regulation
HOP1	YIL072W	DNA binding
IME2	YJL106W,SME1	activation of meiosis
IVY1	YDR229W	phospholipid binding protein
JIP4	YDR475C,YDR474C	unknown function
KAR9	YPL269W	correct positioning of mitotic spindle

Gene	Alt name	Function
LCB4	YOR171C	synthesis of long-chain base phosphates
LDB18	YLL049W	unknown function
LEU3	YLR451W	regulates genes inv in branched chain aa
LHS1	YKL073W,CER1,SSI1	inv in polypeptide translocation and folding
LSB1	YGR136W	inv in actin assembly and polymerization
MCH2	YKL221W	inv in transport of monocarboxylic acids
MCM21	YDR318W,CTF5	minichromosome maintenance
MES1	YGR264C	methionly-tRNA synthetase
MIA40	YKL195W,FMP15,TIM40	inv in import & assembly of intermembrane pr
MNN4	YKL201C	inv in glycosylation
MRC1	YCL061C,YCL060C	inv in replication checkpoint
MRP51	YPL118W	interacts w/ mutations in COX2 & COX3
MSC1	YML128C	unknown function
MSH5	YDL154W	facilitates crossovers b/w homologs
MRS6	YOR370C,MSI4	Rab escort protein
MST28	YAR033W	inv in vesicle formation
MUC1	YIR019C,FLO11,STA4	reqd for pseudohyphal formation
NAB2	YGL122C	reqd for poly(A) tail length control
NDD1	YOR372C	expression of late S-phase specific genes
NFT1	YKR103W	transporter of multi-drug resistant protein
NGR1	YBR212W,RBP1	RNA binding prot,neg regulates growth rate
NMD2	YHR077C,IFS1,UPF2	inv in telomere maintenance
NPP1	YCR026C	nucleotide-triphosphatase activity
NRP1	YDL167C	unknown function
NUP133	YKR082W,RAT3	DNA metabolism
OAC1	YKL120W	mitochondrial inner membrane transporter
OAC4	YCR095C	reqd for Brome mosaic virus replication
OSW1	YOR255W	unknown function
OTU2	YHL013C	unknown function
PAN5	YHR063C	part of panthothenic acid pathway
PBP4	YDL053C	Pbp1p binding protein
PDC1	YLR044C	pyruvate decarboxylase
PET10	YKR046C	unknown function,likely inv in resp growth
PET191	YJR034W	reqd for assembly of cytochrome c oxidase
PFA4	YOL003C	palmitoyltransferase activity
PHD1	YKL043W	enhances pseudohyphal growth,reg FLO11exp
PPA2	YMR267W,IPP2	reqd for mitochondrial function
PPH21	YDL134C,PPH1	inv in signal transduction, regulates mitosis
PPR2	YGL043W,DST1	transcription elongation factor activity
PRC1	YMR297W,LBC1	reqd for protein degradation
PRY1	YJL079C	unknown function
PTC3	YBL056W,	role in DNA checkpoint inactivation
PUS9	YDL036C	mitochrondrial tRNA pseudouridine synthase
PYC2	YBR218C	pyruvate carboxylase
RAD1	YPL022W,LPB9	subunit of Nucleotide Excision Repair factor 1
RAD55	YDR076W	inv in recombinational repair of ds breaks

Gene	Alt name	Function
RDN25-2	RDN25,Rrna	structural constituent of ribosome
REV7	YIL139C	inv in DNA repair,reqd for mutagenesis
RFC4	YOL094C	contributes to DNA clamp loader activity
RLI1	YDR091C	reqd for ribosome biogenesis & translation
RMD11	YHL023C	protein reqd for sporulation
ROG1	YGL144C	lipase activity
RPL12B	YDR418W	structural constituent of ribosome
RPO21	YDL140C,RPB1	DNA-directed RNA polymerase activity
RRM3	YHR031C,RTT104	DNA helicase
SAM1	YLR180W,ETH10	methionine adenosyltransferase activity
SDC25	YLL016W	pseudogene, Ras protein signal transduction
SEC15	YGL233W	Golgi to plasma membrane transport
SEC6	YIL068C	Golgi to plasma membrane transport
SFG1	YOR315W	reqd for growth of superficial pseudohyphae
SIR4	YDR227W,ASD1,UTH2	structural constituent of chromatin
SMC4	YLR086W	ATPase activity
SMY2	YBR172C	unknown function
SNF3	YDL194W	plasma membrane glucose sensor
SNT2	YGL131C	DNA binding protein
SOR1	YJR159W,SDH1	sorbitol dehydrogenase
SPE1	YKL184W,ORD1,SPE10	ornithine decarboxylase
SPE4	YLR146C	spermine synthase
SPO12	YHR152W	nucleolar prot of unknown function
SPO71	YDR104C	reqd for spore wall formation
SPP2	YOR148C	promotes first step of splicing
SRB8	YCR081W,GIG1,NUT6,MED12	subunit of RNA polymerase II
SRV2	YNL138W,CAP	adenylate cyclase binding
STU2	YLR045C	microtubule associated protein
SUT2	YPR009W	putative transcription factor
SWE1	YJL187C,WEE1	protein kinase activity
SWH1	YAR042W,OSH1	phosphatidylinositol binding
SWI1	YPL016W,ADR6,LPA1	chromatin remodeling, transcription factor
SYF1	YDR416W,NTC90	inv in pre-mRNA splicing
TEA1	YOR337W	Ty1 enhancer activator
TELO8P		
THI13	YDL244W	thiamin biosynthesis
THI21	YPL258C	thiamin biosynthesis
TIM11	YDR322C-A,ATP21	ATP synthesis
TMA17	YDL110C	unknown function
TOM1	YDR457W	regulates transcriptional coactivators
TPM2	YIL138C	binds to and stabilizes actin filaments
TPO5	YKL174C	polyamine transporter activity
TRP1	YDR007W	inv in amino acid metabolism
TRX1	YLR043C,LMA1	cytoplasmic thioredoxin isoenzyme
TSC11	YER093C,AVO3	inv in sphingolipid metabolism
TUB2	YFL037W,ARM10,SHE8	structural constituent of cytoskeleton
UBP11	YKR098C	ubiquitin-specific protease
URM1	YIL008W	ubiquitin-like protein

Gene	Alt name	Function
UTP22	YGR090W	snoRNA binding
VBA2	YBR293W	permease of basic amino acids
VMA2	YBR127C,VAT2	ATPase activity
YAL066W		hypothetical protein
YAR009C		DNA directed DNA polymerase activity
YARCTy1-1		retrotransposon
YBL104C		hypothetical protein
YBR012W-B		retrotransposon
YBR204C		serine hydrolase
YBR259W		unknown function
YCR095C	OCA4	reqd for replication of Brome mosaic virus
YDR066C		hypothetical protein
YDR066W		hypothetical protein
YEL077C		hypothetical protein
YFR044C		unknown function
YGL059W		hypothetical protein
YGL081W		hypothetical protein
YHRCTy1-1		retrotransposon
YIL171W-A		ORF, dubious
YIR024C		unknown function
YIR035C		hypothetical protein
YKL044W		hypothetical protein
YLR040C		unknown function
YLR154C-G		unknown function
YLR307C-A		unknown function
YMR181C		unknown function
YNL321W		unknown function
YKL126W	SLI2,YPK1	serine/threonine protein kinase

Task 4 – Characterize Genes of Interest

a. Make heterozygous nulls of genes of interest in non-mutagenized background and measure chromosome loss rate to check reproducibility of phenotype.
b. Characterize the genes and test RAD9 dependence, effects on DNA damage sensitivity and checkpoint response.
c. Look for homologs in mammalian cells and characterize as modifiers of BRCA1-/- mutations in collaboration with ongoing genetic epidemiology studies

Progress:

Task 4a: Gene Ontology (GO) analysis was utilized to help create a list of genes to pursue further. By characterizing the percentage of genes from our screen versus the expected percentage found in the yeast genome GO annotations allow for identification of statistically significantly over- or under-represented groups. In our screen we found that genes involved in the cell cycle, meiosis or localized to the chromosome were significantly over-represented. We thus chose all of the genes from these groups as well as genes that would not have been found from prior studies including essential genes and hypothetical ORFs as our group of 67 genes-of-interest. Recreation of a clean heterozygous deletion of the gene (as opposed to an insertional mutant) was created in both rad9-/- and wt backgrounds for further analysis.

Fluctuation Analysis was performed twice on each of 134 (67 genes in two backgrounds) heterozygous mutants. The mutation rate was then compared to the insertional mutant strains to check for reproducibility of this phenotype. It was found that 89.5% of recreated clean knock-outs, in the rad9-/- background, have the same or greater mutation rate than the insertional mutation; 17.5% of this group have a greater impact on mutation rate in the clean background likley due to the complete deletion having a greated impact on the gene then an insertional mutant. Conversely, 10.5% of recreated clean knock-outs do not repeat the phenotype, possibly due to a second transposon insertion in the original mutant. Southern blots for transposon number are currently in this small number of strains.

Task 4b: Mutation rates in the wt and rad9-/- background were compared in order to determine Rad9 dependence of the increased genome instability. Surprisingly, the majority of mutants had an impact on both the wild type and rad9-/- mutant backgrounds. Only 7 of 67 mutant genes tested were rad9 dependent for demonstrating an increased mutation rate. Of the remaining 60 genes, 36 have a greater than 2 fold increase in mutation rate compared to their rad9-/- or wt parental counterpart. These appear to represent a group of genes that are able to impact genome instability in a heterozygous state.

Semi-quantitative DNA damage assays utilizing exposure to MMS, HU, UV, Benomyl and Phleomycin were performed for this group of 67 mutant strains. The majority of heterozygous strains did not show increased sensitivity to these agents consistent with our prior assays focusing on spontaneous chromosome loss. A group of 21 mutant strains showed either increased sensitivity or resistance to one or more of these agents. These studies are being repeated to confirm these results.

Task 4c: Extensive homology and literature searches are underway to identify which of the 171 genes have human homologs or have homologous pathways in human cells. These genes with create a preliminary list of candidate modifier loci to be tested as part of ongoing genetic epidemiology studies. Professor Tim Rebbeck of the University of Pennsylvania has agreed to include polymorphisms from genes on this list in his ongoing epidemiology studies to identify second site genetic modifiers which impact the age of onset of cancer in women who carry *BRCA1* or *BRCA2* mutations.

Task 5 - Writing of manuscripts and final report.

Progress:

As defined in the reportable outcomes two formal abstracts and poster presentations of this work have been presented. A poster on this work was presented at the annual AACR meeting in Anaheim, CA in April, 2005 as well as at the AACR Cancer Susceptibility and Cancer Susceptibility Syndromes meeting in Maui, HI in March 2006.

Writing of a scientific paper is underway and will be completed when analysis of the molecular mechanisms underlying the increased chromosome loss rate of a subset of genes is completed. An initial Final Report was submitted to the DOD on December 31, 2004. This additional Final Report was prepared as work on this project continued under a no cost extension during 2005. Given the importance of the project the PI has continued to complete this project using institutional funds and has submitted grant applications to the National Cancer Institute to continue this research.

Key Research Accomplishments

(1) Completion of the screening of 30,000 insertional mutatnts for effects on sectoring rate and identification of a group of with consistent genome instability.
(2) Fluctuation Analysis generated mutation rates for all unique insertion sites identified as well as mutation rate calculations of 67 complete gene deletions in rad9-/- and wt backgrounds.
(3) DNA damage assay results of all 67 heterozygous mutants in both rad9-/- and wt backgrounds.

Reportable Outcomes

Insertional mutagenesis coupled with efficient fluctuation analysis allows identification of heterozgyous mutations that impact the chromosome loss rate of a rad9Δ/Δ mutant strain.

Strome E. D. and Plon, S. E. Utilizing Budding Yeast to Identify Potential Modifiers of BRCA1 Function [abstract]. Presented at: Annual Meeting of the American Association for Cancer Research; April 19 2005; Anaheim CA.

Strome E.D. and Plon S. E. Utilizing Budding Yeast to Identify Potential Modifiers of BRCA1 Function [abstract]. Presented at: AACR Cancer Susceptibilty and Cancer Susceptibility Syndromes Meeting March 1-5th 2006; Maui, HI

Conclusions

The goal of this Exploratory Award was to investigate the novel hypothesis that we could develop a methodology to identify heterozygous mutants that impact the genomic stability of a rad9 mutant strain. We have completed this goal and have developed a protocol that allows efficient screening of the yeast genome for such mutations. Analysis of the mutant strains identified in this screen including analysis of the gene ontology of the isolated mutants and quantitative measurement of mutation rate demonstrate that the screen is able to identify heterozygous mutations that impact genomic stability in budding yeast. Analysis of the mutants obtained will allow us to identify a set of genes which are candidates for modifiers of BRCA1 function. Professor Timothy Rebbeck of the University of Pennyslvania has expressed interest in using these loci in his ongoing epidemiology study to identify genetic modifiers that impact that age of onset of breast cancer in women who carry mutations in BRCA1.

References

1. Rosen EM, Fan S, Pestell RG, Goldberg ID: *BRCA1* Gene in Breast Cancer. Journal of Cellular Physiology 196: 19-41, 2003.

2. Zhou BB, Elledge SJ: The DNA damage response: putting checkpoints in perspective. Nature 408: 433-439, 2000.

3. Weinert TA, Hartwell LH: The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science 241: 317-322, 1988.

4. Klein, HL: Spontaneous Chromosome Loss in Saccharomyces cerevisiae Is Suppressed by DNA Damage Checkpoint Functions. Genetics 159: 1501-1509, 2001.

tects replication fork integrity. Relatively little is known, however, how cells that have acquired intolerable damage during replication stress are eliminated and whether this is a function of the DNA replication checkpoint. To explore the role of the DNA replication checkpoint in replication stress-induced apoptosis, we treated human U-2 OS osteosarcoma cells, which have bona fide intact DNA damage checkpoints, with a one-hour pulse of 10 mM hydroxyurea (HU) to deplete the cellular dNTP pool and induce replication fork stalling. Cells were found to transiently downregulate DNA replication and to activate a DNA replication stress response with maximum upregulation of phosphorylated CHK1, BRCA1, and p53, respectively, immediately after the HU pulse. However, induction of apoptosis was a late event and did not occur until 48 hours after HU had been removed. The apoptotic response to HU was preceded by hyperphosphorylation of the retinoblastoma tumor suppressor protein (pRB) at six hours after the HU pulse indicating cell cycle re-entry. In addition, we found that both gamma-H2AX and phosphorylated ATM (Ser 1981) were upregulated beginning 24 hours after ending the HU treatment, raising the possibility that DNA double strand breaks are generated before cells undergo apoptosis. Collectively, our results show that the apoptotic response following transient replication stress involves restarting of the cell cycle machinery and is preceded by a DNA double strand break response. Our results highlight the importance of checkpoint cooperation to maintain genome stability and suppress tumorigenesis.

#3545 Utilizing budding yeast to identify potential modifiers of BRCA1 functions. Erin D. Strome and Sharon E. Plon. *Texas Children's Cancer Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX.*

70% of breast cancers exhibit aneuploidy, and this phenotype is used as an early prognostic factor because it usually indicates a high malignancy potential. Mutations in many different checkpoint genes result in aneuploidy and an increased risk of breast cancer including, *BRCA1, PTEN, CHEK2,* and *ATM*. However, the age of onset and type of cancer developed can vary among women with these mutations, which is partially attributed to the likelihood of the presence of unlinked genetic loci, which modify this risk. We have proposed to identify candidate modifier loci that impact genomic stability by screens in *Saccharomyces cerevisiae*. Budding yeast are commonly used organisms for modeling human processes, and many aspects of the DNA damage checkpoint pathway, in which BRCA1 is involved, are conserved between *S. cerevisiae* and humans. The *S. cerevisiae* Rad9 protein has similar functions and sequence motifs as BRCA1. We therefore developed a screen based on the hypotheses that *rad9*Δ/Δ mutations in *S. cerevisiae* will model *BRCA1-/-* mammary cells and haploinsufficiency at a second locus that alters the chromosome loss rate of our *rad9*Δ/Δ diploid strains serves as a potential modifier loci. To complete the screen we created a *rad9*Δ/Δ yeast strain carrying two marker systems for chromosome stability assays allowing a visible qualitative marker and a canavinine resistance quantitative marker of chromosome loss rate by fluctuation analysis. We utilize an insertional mutagenesis library to screen the yeast genome for heterozygous mutations, which alter chromosome loss rates. To date we have screened 7500 insertional mutants for genomic instability and qualitatively identified 10 candidates, 5 of which alter the chromosome loss rate of the *rad9*Δ/Δ yeast strain by ~10 fold. The insertions include disruption of uncharacterized yeast ORFs as well as a gene implicated in chromatin silencing, which will need be further characterized via directed mutation of these genes and analysis for radiation sensitivity, DNA damage checkpoint function and genomic instability. Thus, our screening strategy is capable of identifying heterozygous mutations that alter genomic stability and the human homologs of genes identified in this screen can serve as candidate modifiers of genomic stability in mammary cells null for BRCA1 function. Funding thanks to: U.S. Army Breast Cancer Research Grant.

#3546 Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage. Wenya Huang, Yi-Hsuan Hsieh, Ih-Jen Su, Hui-Ching Wang, Wen-Wei Chang, Huan-Yao Lei, Ming-Derg Lai, Wen-Tsan Chang, Wen-Tsan Chang. *National Cheng Kung University, Tainan, Taiwan Republic of China and National Health Research Institute, Tainan, Taiwan Republic of China.*

Ground glass hepatocytes (GGHs) are the historic hallmarks for the hepatocytes in the late and nonreplicative stages of hepatitis B virus (HBV) infection. We have identified type I and type II GGHs, which contain two mutant types of large HBV surface antigens (HBsAg) with deletions over the pre-S_1 and pre-S_2 regions, respectively. These pre-S mutant HBV surface antigens accumulate in endoplasmic reticulum (ER), resulting in strong ER stress. Type II GGHs often appear in hepatic nodules in the late phases of HBV infection and proliferate in clusters, suggesting that these mutant pre-S_1/S_2 HBsAg may be involved in HBV-related hepatocarcinogenesis, associated with ER stress. In this study, we investigated the potential genomic instability imposed by pre-S mutant HBsAg. Based on the analysis of comet assays, we found that the pre-S_1 and pre-S_2 mutant HBsAg caused oxidative stress and DNA damage. The DNA repair gene *ogg1* was greatly induced by overexpression of pre-S mutant HBsAg. Induction of the DNA repair gene *ogg1* was also detected in the pre-S_2 HBsAg transgenic mice, as well as the type II GGHs from patients with hepatocellular carcinoma, strongly suggesting that the pre-S mutant HBsAg contributes to the oxidative DNA damage to hepatocytes. In addition, the mutation rates in the X-linked *hprt* gene were enhanced in mouse hepatoma ML1-4a cells, which constitutively expressed the pre-S_1/S_2 HBsAg. These results indicate that pre-S_1/S_2 mutant HBsAg, which make up GGHs, induce oxidative DNA damage and mutations in hepatocytes in the late stages of HBV infection.

#3547 Development of an internal standard for the Comet assay: Minimizing intra- and inter-experiment variability in the measures of DNA damage formation and repair. Murizal Zainol, Gabriela M. Almeida, Benedict T. Sherwood, Karen J. Bowman, George D. D. Jones. *Institute of Medical Research, Kuala Lumpur, Malaysia and University of Leicester, Leicester, United Kingdom.*

The Comet assay (also known as single cell gel electrophoresis (SCGE)) is a highly sensitive, rapid and straightforward fluorescent microscopy method for assessing both DNA damage formation and repair at the level of individual cells. Furthermore, due to its ability to detect a wide variety of DNA damage in eukaryotic cells exposed to a range of genotoxins, its ease of application, and both its low cost and low material requirement, the technique is being increasingly exploited in human biomonitoring studies. However, many features of the assay, particularly of the electrophoresis stage, effect both intra-assay variability and inter-assay reproducibility. To minimise these variables, we have designed an internal standard for the Comet assay consisting of 'reference' cells which have had their DNA thymidine substituted with BrdU. The post-electrophoresis comets derived from these 'reference' cells can be readily distinguished from the 'test' cell comets present in the same gel, at the time of comet analysis using a fluorescently tagged *anti*-BrdU antibody together with an appropriate dichoric mirror and barrier filter set to selectively visualise and distinguish the 'test' and 'reference' cells. In dose response experiments, the 'reference' and 'test' cells were either present in separate gels (on the same slide) or mixed together in the same gel before their co-exposure (on slides) to X-irradiation. By adjusting the individual 'reference' cell data to a determined average 'reference' cell response and then applying the derived correction factor to the corresponding individual 'test' cell data, we have obtained substantial (>2-fold) reductions in the coefficient of variation (CoV) for repeated measures of radiation-induced comet formation and DNA damage repair, but only when the 'reference' and 'test' cells were in the same gel; only minor reductions in CoV were noted when the 'reference' and 'test' cells were in separate gels. These studies indicate that differences between individual gels, even when present on the same slide, significantly contribute to experimental variation in the Comet assay. Having both the 'reference' and 'test' cells together in the same gel provides the means of reducing variation in comet measures caused by differences in the preparation of the slides, cell exposure, nucleoid electrophoresis and comet analysis. Ultimately, we anticipate that this research will deliver 'off the shelf' quality assurance materials for investigators using the Comet assay.

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#3548 Genomic profiling of familial pancreatic cancer by combination of laser capture microdissection and whole genome amplification. Tadayoshi Abe, Noriyoshi Fukushima, Norihiro Sato, Hiroyuki Matsubayashi, Gloria Petersen, Ralph H. Hruban, Michael Goggins. *Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD and Mayo Clinic, Rochester, MN.*

Approximately 10-20 percent of patients with pancreatic cancer have a significant inherited predisposition to developing the disease. Familial pancreatic cancer (FPC) is defined as family having at least two first-degree relatives with pancreatic cancer that do not fulfill the criteria of another hereditary cancer syndrome. Ten to 20% of inherited PC is associated with germline mutations in the BRCA2, p16, PRSS1, STK11, FANCC or mismatch-repair genes. The identification of familial pancreatic cancer susceptibility genes is challenging because conventional familial genetics studies such as linkage analysis is difficult since affected patients from FPC families usually die of their disease rapidly and are unable to contribute to research studies. To compliment linkage studies, we are performing molecular genetic studies of familial pancreatic cancers to identify loci specifically targeted for losses in these neoplasms. Recently, an isothermal whole genome amplification method using the strand-displacing ø29 polymerase was developed and successfully applied to amplify the small amount of DNA. This method, termed "whole genome multiple strand displacement amplification (MDA)," demonstrated a high-amplification

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Utilizing Budding Yeast to Identify Potential Modifiers of BRCA1 Function

Erin D. Strome, Sharon E. Plon. Baylor College of Medicine, Houston, TX.

Seventy percent of breast cancers exhibit aneuploidy, and this phenotype is used as an early prognostic factor because it usually indicates a high malignancy potential. Inherited mutations in many different checkpoint genes result in aneuploidy and lead to an increased risk of breast cancer including, BRCA1, PTEN, CHEK2, and ATM. However, the age of onset and type of cancer can vary among mutation carriers and this difference is at least partially attributed to the presence of unlinked genetic loci, which modify this risk. We decided to identify candidate modifier loci that impact genomic stability utilizing yeast genetics; based on the hypotheses that rad9-/- mutations in S. cerevisiae will model BRCA1-/- mammary cells and haploinsufficiency at a second locus that alters the chromosome loss rate of a rad9-/- diploid strain serves as a potential modifier locus. To carry out such a screen, we created a rad9-/- yeast strain carrying two marker systems for chromosome stability assays sensitive enough to detect the impact of heterozygous mutations. The first assay measures sectoring of a colorimetric marker based on chromosome fragment loss and the second assay utilizes a canavanine resistance marker to allow quantitative determination of endogenous whole chromosome loss rates by fluctuation analysis (FA). We utilized an insertional mutagenesis library to screen the yeast genome for heterozygous mutations, analyzing 30,000 mutants for 95% coverage. We identified 400 independent insertions that alter chromosome fragment loss rate and are currently finishing identification of the insertion sites and performing FA to determine the quantitative effect of mutations on genomic stability. Recreation of knock-outs, utilizing a KAN cassette, in a wild-type and rad9-/- background is allowing us to both verify the instability phenotype and characterize our mutants as RAD9 dependent or independent. This latter grouping serves to distinguish modifiers of rad9 mutant strains from heterozygous mutations that cause genomic instability on their own. Furthermore we have utilized Gene Ontology (GO) annotations of the preliminary list of genes disrupted by the insertional mutations to identify gene groupings which are significantly overrepresented. These groups include the expected "cell cycle" group but also the unexpected "amino acid and derivative metabolism" biological process group. Consistent with this finding, several other mutants also impact amino acid transport or protein degradation. Hypothesizing that these mutations are affecting pathways sensitive to amino acid levels, (such as the TOR pathway), we are further characterizing these heterozygous mutant strains for amino acid concentrations. This work will lead to further investigation of the impact of these pathways on genomic instability in yeast and subsequently on mammalian cells.